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(54) Title: A METHOD FOR THE AMPLIFICATION OF MULTIPLE GENETIC TARGETS

(57) **Abstract:** This invention provides universal method for simultaneous amplification and detection of multiple genetic targets. The method provided facilitates simultaneous amplification of multiple genetic targets, without requiring optimization of the reaction conditions. Also provided are kits containing reagents necessary for carrying out the universal amplification method. The primer pairs are at a final concentration of approximately 0.0025 µM - 0.05 µM per primer and Mg^{2+} at a concentration of approximately 5 - 12.5 mM in the reaction mixture.

WO 2004/040013 A1

A METHOD FOR THE AMPLIFICATION OF MULTIPLE GENETIC TARGETS

TECHNICAL FIELD

This invention relates to a method for the amplification and detection of multiple genetic targets, and components thereof. More specifically, the present invention relates to the simultaneous amplification of multiple genetic targets in a single reaction. In particular, the present invention relates to an improved multiplex Polymerase Chain Reaction (PCR) method and components thereof.

BACKGROUND OF THE INVENTION

The Polymerase Chain Reaction (PCR) is a widely used technique that employs a thermostable DNA polymerase enzyme in conjunction with target-specific primers to amplify target genetic sequences. The term "multiplex" refers to the ability to amplify multiple genetic sequences simultaneously, in a single reaction vessel, rather than having to conduct each amplification reaction individually. Thus, multiplexing reactions saves time and resources. Multiplex PCR generally refers to a multiplex reaction using standard PCR reagents and unmodified deoxyribonucleotide triphosphates (dNTPs) for amplifying multiple genetic sequences simultaneously. In the original PCR patents, U.S. Patent Nos. 4,683,195 and 4,683,202, Mullis contemplated the amplification of "at least one" target sequence and suggests that multiple targets may be amplified. Mullis, however, failed to teach successful multiplex PCR and all of the examples of the original patents disclose amplification of a single target sequence. In practice, it has been found that multiplex PCR requires different experimental conditions than singleplex PCR.

U.S. Patent No. 5,582,989 discloses one of the first uses of multiplex PCR to detect clinically relevant DNA sequences specific for Duschene's Muscular Dystrophy (DMD). Certain claims of this patent require that the lowest Tm and highest Tm of all added primers vary by no more than 8.3 or 4.4°C. Further, during prosecution of the patent, the applicant argued that a critical requirement for successful multiplex PCR is carefully balanced primer compositions. Specifically, the applicant stated that the primer pairs used in multiplex PCR must be selected to have similar annealing (or melting) temperatures. It is clear that the need for all of the primers to have similar annealing

temperatures restricts the utility of multiplex PCR. Moreover, as indicated in U.S. Patent No. 5,582,989, the use of primer pairs having similar melting temperatures did not obviate the need to optimise each reaction in order to ensure simultaneous amplification of target sequences. According to the patent, reaction conditions may need to be modified depending on the particular regions to be amplified, the number and length of the sequences to be amplified and the choice of oligonucleotide primers.

The experimental conditions taught in U.S. Patent No. 5,582,989 were publicly disclosed by Chamberlain et al. (1988) *Nucleic Acids Res.*, 16(23):11141-56. Since that time, researchers continue to empirically optimize multiplex PCR conditions in order to successfully amplify multiple targets simultaneously.

Researchers have attempted to optimize a large number of variables in order to develop successful multiplex PCR (See, for example, Markoulatos et al. (2002) *Journal of Clinical Laboratory Analysis*, 16:47–51; Henegariu et al., (1997) *Biotechniques*, 23(3):504-11; and http://info.med.yale.edu/genetics/ward/tavi/Guide.html). Examples of variables that are routinely optimized include primer melting temperatures, primer concentration, concentration of Tris-HCl, NH_4^+, and K^+ in the reaction buffer, $MgCl_2$ concentration, annealing time and temperature, extension time and temperature, touchdown PCR (if used), type and concentration of DNA polymerase enzyme, dNTP concentration and possible interaction with magnesium, and additives like BSA, glyercol and other synthetic agents. There is no current method or procedure that indicates which, if any, of these variables are critically important for successful multiplex PCR. Thus, different researchers use different combinations of variables.

By way of example, Markoulatos, et al. (2001) *Journal of Clinical Microbiology*, 39(12): 4426-4432, taught the use of 10 pmol of each primer in a final volume of 50 µl, a final concentration of 0.8 mM each dNTP, and a final $MgCl_2$ concentration of 2 mM. Kariyama et al. (2000) *Journal of Clinical Microbiology*, 38(8): 3092-3095, taught the use of primers ranging in amount from 1.25-5 pmol in a final volume of 25 µl, 0.2 mM each dNTP, and a final $MgCl_2$ concentration of 1.5 mM.

Lem et al. (2001) *Diag. Microbiol. Infect. Disease* 41:165-168 describe a 3plex assay using multiplex PCR conditions including touchdown PCR, a hotstart DNA polymerase enzyme, a final $MgCl_2$ concentration of 9 mM and a final primer

concentration of 0.017 μM (or 0.51 pmol). However, this reference does not teach or suggest a universally applicable multiplex PCR amplification of more than 3 target sequences using any pre-selected set of primers.

It is both tedious and time-consuming to optimize all, or a portion, of the possible variables of multiplex PCR each time a new combination of primers or different target sequences are used. Even following optimization, with previous methods there has been no certainty that a given primer pair would function in the presence of additional primers in a multiplex PCR assay, irrespective of the fact that a given primer pair is known to work effectively in singleplex reactions. Typically, the addition or removal of a primer pair in an existing working assay required the optimization process to begin all over again. There remains a need for a universal set of multiplex PCR reaction conditions that do not require optimization each time a different primer is added or removed from the assay.

U.S. Patent Nos. 5,882,856 and 6,207,372 relate to a universal primer sequence for multiplex DNA amplification. In particular, these patents disclose chimeric primers that serve as high stringency recognition sequences in the amplification process and normalize the degree of amplification of different targets. Although these primers are described as having a uniformly high degree of specificity in the annealing reactions that occur between different primers present in the mixture and their cognate target sequences in the DNA template without requiring the need to adjust multiplex reaction conditions, the design of these primers is both complex and time consuming. Clearly, a technician could not readily design these primers for use in a DNA amplification protocol, nor replace a given set of primers in the midst of an amplification assay.

U.S. Patent No. 6,333,179 relates to methods and compositions for multiplex amplification of nucleic acids. According to this patent, a predetermined ratio of primers can be calculated according to the disclosed formula to achieve an approximately equi-molar yield of multiplex PCR products. According to this formula, primer concentrations are varied as a function of amplicon length. Such a method is time-consuming, requiring individual calculations for each primer pair. It would be desirable to have a method for simultaneously amplifying multiple genetic targets that could be

repeatedly performed according to a set of common directions without requiring optimization of the reaction conditions.

Qiagen has developed and is currently marketing a multiplex PCR kit that is purported to eliminate the need for optimization. The kit is provided with a master mix that contains HotStarTaq™ DNA polymerase, $MgCl_2$, dNTPs and a PCR buffer pH 8.7. The final concentration of $MgCl_2$ in the reaction is 3 mM and the recommended final primer concentration within a range of 0.1 to 0.3 μM (5 pmol to 15 pmol), preferably 0.2 μM (10 pmol). The reaction buffer is said to contain a special balance of NH_4^+ and K^+ cations to allow for successful multiplex PCR. Furthermore, the kit includes a "Q-solution," which contains a PCR additive designed to facilitate amplification of difficult templates by modifying the melting behaviour of DNA. If the multiplex PCR does not work effectively, the user is advised to optimize the reaction using the Q-solution.

In many cases it is desirable to be able to simultaneously amplify numerous genetic targets in a convenient and cost-effective manner. In the field of infectious disease, for example, often a practitioner is interested in pinpointing a causative agent of infection from a large group of potential organisms. It would be beneficial to have a multiplex PCR system capable of simultaneously amplifying more than 3 target genetic sequences without the need for optimization steps.

In summary, there remains a need for a universally applicable method for efficiently and economically amplifying multiple genetic targets in a single reaction vessel, without the need for optimization of multiplex PCR reaction conditions.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a universal multiplex PCR method for simultaneously amplifying multiple genetic targets in a single reaction vessel.

It is a further object of the present invention to provide a universal multiplex PCR method for simultaneously amplifying multiple genetic targets in a single reaction vessel without requiring optimization of the reaction conditions.

It is another object of the present invention to provide a novel PCR reaction mixture adaptable for simultaneously amplifying multiple genetic targets in a single reaction vessel.

It is yet a further object of the present invention to provide a kit adaptable for simultaneously amplifying multiple genetic targets in a single reaction vessel.

A universally applicable multiplex PCR method for simultaneous amplification of multiple genetic targets is provided. According to the present invention, multiple genetic targets can be quickly and easily detected without requiring extensive optimization of the universal multiplex PCR method herein described. The method of the present invention is pre-optimized for amplification of multiple genetic targets and can be performed without the need for optimization of the multiplex PCR reaction conditions.

According to one aspect of the present invention, there is provided a universal method for simultaneously amplifying greater than three genetic targets in a sample at equivalent amplification efficiencies, said method comprising: (i) providing a primer pair specific to each of said greater than three genetic targets; (ii) adding said primer pairs to a PCR mixture; (iii) performing a series of PCR steps to amplify each of said multiple genetic sequences in said sample; (iv) wherein said PCR reaction mixture comprises said primer pairs at a final concentration of approximately 0.0025 μM – 0.05 μM per primer and Mg^{2+} at a concentration of approximately 5 – 12.5 mM in the reaction mixture.

According to another aspect of the present invention, there is provided a method for preparing a PCR mixture for simultaneously amplifying greater than three genetic targets at equivalent amplification efficiencies, said method comprising: (i) providing a PCR buffer or sterile water for dilution of PCR reactants; (ii) adding deoxyribonucleotides to the PCR buffer or water; (iii) adding Mg^{2+} to the PCR buffer or water such that the final concentration of Mg^{2+} in the PCR mixture is between 5.0 and 12.5 mM; and (iv) adding a primer pair for each of said greater than three genetic targets to the PCR buffer or water such that the final concentration of each primer concentration to between 0.0025 and 0.05 μM.

According to another aspect of the present invention, there is provided a method for simultaneously detecting greater than three genetic targets in a sample, said method comprising: (i) providing a primer pair specific to each of said greater than three genetic targets; (ii) adding said primer pairs to a PCR mixture; (iii) performing a series of PCR steps to amplify each of said greater than three genetic sequences in said sample; and (iv) detecting amplicons produced from the series of PCR steps, wherein said PCR reaction mixture comprises said primer pairs at a final concentration of approximately 0.0025 μM – 0.05 μM per primer and Mg^{2+} at a concentration of approximately 5 – 12.5 mM in the reaction mixture.

According to another aspect of the present invention, there is provided a PCR mixture for use in simultaneously amplifying greater than three genetic targets at equivalent efficiencies, said mixture comprising a PCR buffer or water containing dNTPs, 5.0 – 12.5 mM Mg^{2+} and a primer pair for each of said greater than three genetic targets at a concentration of 0.0025 – 0.05 μM, wherein said PCR mixture in combination with a thermophilic DNA polymerase is suitable for simultaneously amplifying the greater than three genetic targets in a single reaction vessel without optimization.

According to another aspect of the present invention, there is provided a kit for simultaneously amplifying greater than three genetic targets for detection, said kit comprising:

- a PCR solution comprising Mg^{2+} and dNTPs; and
- a primer solution comprising a primer pair corresponding to each of said greater than three genetic targets; and
- a set of instructions for using contents of said kit to produce a PCR mixture for simultaneously amplifying the greater than three genetic targets in a sample to be tested,

wherein said PCR solution contains sufficient Mg^{2+} to produce a final concentration of 5.0 – 12.5 mM in the PCR mixture and said primer solution contains sufficient primers to produce a final concentration of 0.0025 – 0.05 μM of each primer in the PCR mixture.

BRIEF DESCRIPTION OF THE DRAWINGS

Further features and advantages of the present invention will become apparent from the following detailed description, taken in combination with the appended drawings, in which:

Figure 1 is a flowchart outlining a universal multiplex PCR method in accordance with one embodiment of the present invention;

Figure 2 illustrates the detection of ten different genes in methicillin-resistant *Staphylococcus aureus* using a universal multiplex PCR method in accordance with one embodiment of the present invention.

Figure 3 illustrates the detection sensitivity of a multiplex PCR of ten different genetic targets as displayed at varying initial concentrations of bacteria in accordance with one embodiment of the present invention;

Figure 4 illustrates the effect of varying magnesium chloride concentrations between 5 mM and 10 mM on the detection of ten different genetic targets in accordance with an embodiment of the present invention;

Figure 5 illustrates the effect of varying magnesium chloride concentrations between 1.5 mM and 15 mM on the detection of ten different genetic targets in accordance with an embodiment of the present invention;

Figure 6 illustrates the effects of varying primer concentrations between 0.0025 and 0.3 μM on the detection of ten different genetic targets in accordance with an embodiment of the present invention;

Figure 7 illustrates the effects of adding additional primer pairs on the detection of genetic targets in accordance with an embodiment of the present invention;

Figure 8 illustrates the effects of primer melting temperature differences on the detection of eight different genetic targets in accordance with an embodiment of the present invention;

Figure 9 illustrates the effects of using different DNA polymerase enzymes on the detection of ten different genetic targets in accordance with an embodiment of the present invention;

Figure 10 illustrates the effects of varying the amount of DNA polymerase enzyme on the detection of ten different genetic targets in accordance with an embodiment of the present invention;

Figure 11 illustrates the detection of ten different genetic targets using a fast multiplex PCR in accordance with an embodiment of the present invention;

Figure 12 illustrates the detection of ten different genetic targets using a fast, two-temperature multiplex PCR in accordance with an embodiment of the present invention;

Figure 13 illustrates the effects of varying the amount of dNTPs on the detection of ten different genetic targets in accordance with an embodiment of the present invention;

Figure 14 illustrates the detection of sixteen different genetic targets using a multiplex PCR in accordance with an embodiment of the present invention;

Figure 15 illustrates the detection of ten different genetic targets using multiplex PCR master mixtures, following storage, in accordance with an embodiment of the present invention;

Figures 16A and B illustrate the detection of five different genetic targets using a multiplex PCR in accordance with an embodiment of the present invention, wherein Figure 16A depicts the results following detection using agarose gel electrophoresis and ethidium bromide staining and Figure 16B depicts the results following capillary electrophoresis and fluorescence detection;

Figure 17 illustrates simultaneous amplification of large genetic targets using a universal 4plex PCR method in accordance with one embodiment of the present invention;

Figure 18 illustrates the results of a standard 10plex PCR in comparison to a universal 10plex PCR in accordance with one embodiment of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

A universally applicable multiplex Polymerase Chain Reaction (PCR) method for reliable and efficient amplification of multiple genetic targets in a single reaction vessel is provided by the present invention. The ability to simultaneously amplify and detect multiple genetic targets using a convenient and time-efficient method of multiplex PCR as taught herein, provides a clear advantage over the prior art. The present invention provides a simple, efficient and economical method for achieving multiple target genetic sequence amplification in a single reaction. In particular, the multiplex PCR method of the present invention is applicable to any combination of target and primer sequences and does not require optimization, the addition of additives or the use of complicated universal primers.

"Amplification" of DNA as used herein denotes the use of polymerase chain reaction (PCR) to increase the concentration of a particular DNA sequence within a mixture of DNA sequences.

An "amplicon" is a product of the amplification of a target genetic sequence.

"Multiplex PCR" as used herein refers to the use of the polymerase chain reaction (PCR) for simultaneous amplification of multiple genetic targets in a single polymerase chain reaction (PCR) reaction. PCR as used herein may include touch-down PCR.

A "PCR reaction mixture" as used herein denotes a mixture, adaptable for simultaneously amplifying multiple genetic targets under suitable conditions for PCR.

A "genetic target" as used herein denotes a genetic sequence capable of amplification by polymerase chain reaction (PCR). A genetic target in accordance with the present invention includes any DNA sequence, including bacterial, viral, human, plant, and animal DNA, for example.

Generally, it is technically more challenging to design a multiplex PCR system that can amplify multiple genetic targets than a standard PCR system for amplification of

a single target sequence. Different primer pairs have different amplification efficiencies, thus making it difficult to achieve adequate amplification of all primer pairs simultaneously. Due to the iterative nature of PCR cycles, amplicons generated by more efficient primer pairs quickly become the dominant species in the mix. This dominant species then out-competes the less efficient primer pairs for PCR reagents, resulting in insufficient amplification of the less efficient species.

The present invention provides a multiplex PCR reaction method that can be used with any pre-selected primer pairs specific for the genetic targets of interest. The improved multiplex PCR reaction method of the present invention includes pre-set amplification reaction conditions. Pre-selected primer pairs of the present invention are target specific primer pairs designed according to any standard primer design protocol. The unique reaction conditions of the present invention serve to optimize the amplification efficiencies of the target specific primer pairs to effectively amplify more than three, genetic targets simultaneously. According to a preferred embodiment of the invention, 16 or more genetic targets can be simultaneously amplified in the same reaction vessel. The present invention can be employed using standard PCR equipment.

In developing a universal multiplex PCR method, it has now been surprisingly found that the use of a final Mg^{2+} concentration of between 5 – 12.5 mM and individual primer concentrations of 0.005 – 0.05 μM in a standard PCR reaction buffer facilitates efficient and effective amplification of target sequences without further optimization of the reaction conditions. New primers can be added or subtracted from the reaction mixture without altering the final Mg^{2+} or final primer concentrations.

PCR Components

Magnesium

Typical PCRs, including multiplex PCRs, make use of a final Mg^{2+} concentration of approximately 1.5 mM, although higher concentrations have been used, for example, in the Qiagen Multiplex PCR kit, which uses a final concentration of 3 mM. Mg^{2+} is necessary as a cofactor for the DNA polymerase enzyme and may also serve to stabilize primer annealing to DNA templates. Higher Mg^{2+} concentrations are generally understood to improve sensitivity at the expense of specificity. For this reason, in the

past, high Mg^{2+} concentrations have been avoided in the development of multiplex PCRs. In contrast to this general understanding, the present invention demonstrates that the use of high Mg^{2+} concentrations does not result in a decrease in specificity of the multiplex PCR provided that the primer concentrations are between 0.005 – 0.05 μM. High Mg^{2+} concentrations used in the multiplex PCR method of the present invention are in the range of 5 – 12.5 mM, or advantageously 5 – 10 mM.

Primers

The primer concentrations used in the method of the present invention are significantly lower than those typically used in standard PCR and multiplex PCR. Standard PCRs typically make use of a final primer concentration of 1 μM each. Multiplex PCRs, such as that marketed by Qiagen, can make use of lower primer concentrations, however, these are still approximately ten fold higher than the concentrations used in the method of the present invention.

The universal multiplex PCR method of the present invention makes use of a primer concentration (each primer) of 0.0025 – 0.05 μM, or advantageously 0.01 – 0.02 μM.

Primer pairs of the present invention are designed according to a primer design protocol or selected according to certain primer selection criteria and employed in the universal multiplex PCR method at a pre-determined concentration of between 0.0025 – 0.05 μM, or 0.01 – 0.02 μM each. The pre-determined concentration of each primer pair in the reaction mixture of the present invention is preferably the same. Furthermore, target-specific primer pairs of the present invention can be added, removed or replaced in the reaction mixture of the present invention without requiring any optimization of the reaction conditions. As provided in accordance with the present invention, the competing efficiency of the primer pairs in the reaction mixture is reduced in comparison to that observed in standard multiplex PCRs, and thus does not result in a need for optimization of the reaction conditions. This aspect of the invention is a significant improvement over the prior art.

The present invention is easily tailored to amplify a preferred number of target genetic sequences without requiring time-consuming optimization steps. For example, in

the event that a sample is scheduled to be screened for nine genetic targets, and it is subsequently determined that an additional genetic target is also of interest, the present invention may be adapted to accommodate the screening of all 10 genetic targets, simultaneously. In doing so, a standard primer design protocol or primer selection criteria would be used to obtain suitable primer pairs for the additional genetic targets of interest. In accordance with the method of the present invention as herein disclosed, these additional primer pairs would be added to the reaction mixture at the predetermined concentration. Likewise, primer pairs can be removed from the reaction mixture of the present invention without disturbing the output of the reaction.

In accordance with another embodiment of the present invention primer pairs can be replaced by alternative primer pairs as designed or selected according to the purpose of the reaction.

In one embodiment of the present invention, once the targets that comprise a multiplex set are determined, primer pairs complementary to each target sequence are designed. This can be accomplished using any of several software products that design primer sequences, such as OLIGO™ (Molecular Biology Insights, Inc., CO), Gene Runner (Hastings Software Inc., NY), or Primer3™ (The Whitehead Institute, MA).

Typically PCR primers are designed to have melting temperatures in the range of 55 to 65°C, however, the inventors have surprisingly found that the method of the present invention does not require all of the melting temperature of primers to be within a defined range. The primers must, however, be designed using standard procedures to ensure that they are capable of hybridizing to the target DNA for amplification. Standardizing the melting temperatures of the primers to within a set range may facilitate the uniformity of the primer hybridization kinetics, however, this is not necessary in order for the method of the present invention to function effectively.

The primers of the present invention may be designed to produce a PCR product or amplicon of virtually any size. In accordance with the present invention a PCR product or amplicon may include a target genetic sequence as amplified and detected in accordance herewith. Typically amplicons of the present invention will range in size from 50 to 2000 base pairs (bp) or nucleotides (nt), or from 100 to 1500 bp. According to a preferred embodiment of the present invention, primers are designed to produce a

PCR product or amplicon that between 100 and 1000 base pairs (bp) or nucleotides (nt) in length. It is fully contemplated that the present invention is adaptable for the amplification of amplicons larger than 2000 bp. In accordance with this embodiment, consideration should be given to the type of enzyme employed in connection with the present invention as well as the means used for detecting the amplicons.

In accordance with another embodiment of the present invention, amplicons are preferably of different lengths. A 20 bp difference in amplicon length is preferred when detection of the amplicons includes agarose gel electrophoresis and ethidium bromide staining. Alternatively, a single base pair difference in amplicon length may also be detected in accordance with the present invention. In this instance, a detection system such as polyacrylamide gel electrophoresis may be employed. It should be understood that the present invention may be employed to amplify genetic targets producing amplicons of any size. Furthermore, the method of the present invention may be employed with a variety of detection means.

In accordance with one embodiment of the present invention, the primers are designed to have a GC content that ranges from 20 to 80%.

Selection of the remaining reaction conditions is not critical to the efficiency and effectiveness of the universal multiplex PCR method of the present invention. Provided that the Mg^{2+} and primer concentrations are within the specified ranges, any standard PCR buffer, thermophilic DNA polymerase and concentration of dNTPs may be employed. The selection of these components is well within the abilities of a worker skilled in PCR and may be based on such considerations as availability, cost and downstream detection methods.

The unique Mg^{2+} and primer concentrations, coupled with target-specific primer pair sequences and a series of thermal cycling conditions provide a universal multiplex PCR system that reliably and efficiently amplifies multiple target genetic sequences simultaneously, in accordance with one embodiment of the present invention.

Deoxyribonucleotides

Standard dNTP concentrations are used in the universal multiplex PCR method of the present invention. Typically, the dNTP concentrations ranges from 0.25 mM to 1.25 mM although the reaction will proceed in the presence of lower dNTP concentrations. More preferably, however, a final dNTP concentration of approximately 0.25 mM is used in a reaction mixture of the present invention. The specific concentration of dNTPs for use in the method of the present invention is not critical provided that the concentration is selected to avoid the possibility of the dNTPs becoming limiting reagents in the PCR.

Enzyme

Any PCR enzyme known in the art may be employed in accordance with the present invention, such as, but not limited to Taq polymerase or Pfu polymerase. Although it is not necessary to use a hot start in the method of the present invention, one embodiment makes use of a hot start enzyme, such as Amplitaq Gold® or HotStarTaq™. Alternatively, in the absence of a hot start enzyme, a manual hot start step may be employed together with a standard PCR enzyme, as known in the art. It is fully contemplated that other enzymes capable of amplifying genetic sequences may be employed in accordance with the present invention and as advances are made in the field of thermophilic DNA polymerases, such improved polymerases may be used in the multiplex PCR method of the present invention.

As indicated above the universal PCR method of the present invention can be performed using any thermophilic DNA polymerase. It is well known that different DNA polymerases have different buffer requirements and different buffer conditions for optimum activity. The selection of the appropriate buffer is well within the abilities of the skilled artisan. Some DNA polymerases have been found to be effective in the universal PCR method of the present invention, even when the enzyme's standard PCR buffer is replaced with water. For example, the use of AmpliTaq Gold® with no buffer and using only water as a diluent was found to be effective in the PCR method of the present invention.

Performing the Reaction

The PCR of the present invention is performed using the same steps as in standard PCR, namely successive cycles of denaturation of double stranded target nucleic acid and annealing and extension of the primers to produce a large number of copies of segments of the target DNA. Each cycle is a thermocycle in which the reaction temperature is raised to denature the double stranded DNA and lowered to allow annealing and extension. As would be understood by a worker skilled in the art, the denaturation, annealing and extension temperatures will vary in different reactions and are selected based on primer sequences, enzyme, degree of specificity required, etc.

PCR techniques applicable to the present invention include *inter alia* those described in "PCR Primer: A Laboratory Manual", Dieffenback, C.W. and Dveksler, G.S., eds., Cold Spring Harbor Laboratory Press (1995); "Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia", Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N, *Science* (1985) Dec 20; 230(4732):1350-4.

In one embodiment of the present invention, the PCR makes use of successive three-step cycles in which the temperature is raised to a first temperature for denaturation of the double stranded DNA, lowered to a second temperature to allow annealing of the primers and raised to a third temperature, which is between the first temperature and the second temperature, for the extension step. In an alternative embodiment of the present invention the PCR makes use of successive two-step cycles in which the temperature is raised to a first temperature for denaturation the double stranded DNA, and lowered to a second temperature allow annealing of the primers and extension of the primers.

In the case of PCR using either a two-step or a three-step cycle, the reaction will make use of more than 20 cycles. In one embodiment of the present invention between 20 and 50 PCR cycles are performed. In a specific embodiment of the present invention, 40 cycles of a two-step cycle are performed in which each two step cycle comprises a denaturation step in which the reaction mixture is heated at 95°C for 1 second and an annealing and extension step in which the reaction mixture is heated at about 50 – 72°C for 10 seconds.

As depicted in Figure 1, the PCR of the present invention optionally includes a hot start initiation. Hot start initiation of PCR is well known in the art and may be performed by mixing all of the reagents for PCR, except for either the enzyme or the primer mixture, heating the mixture to the denaturation temperature and subsequently adding the enzyme or primer mixture that was left out of the mixture.

In accordance with one embodiment of the present invention the PCR includes touchdown PCR. Touchdown, or step-down PCR, refers to incremental decrease of the annealing temperature with each cycle. If touchdown PCR is employed, 15 to 20 cycles of touchdown PCR may be performed prior to the standard PCR cycles. It is fully contemplated that the PCR steps of the present invention may include steps of standard PCR alone, or in combination with touchdown PCR.

Optionally, the PCR includes a final elongation step following completion of the standard PCR cycles. Typically, if included, the final elongation is performed at 72°C for approximately 7 minutes.

Once the reaction is complete the mixture is cooled to approximately 4°C. At this point the mixture may be stored or used immediately in a detection method.

Detection

Following amplification, the amplicons may be detected using any method known in the art, including, without limitation, gel electrophoresis in agarose or acrylamide gels; real-time detection; non-isotopic calorimetric detection; chemiluminescent, and fluorescent detection. Detection may be quantitative or qualitative depending on the techniques used. Generally, in the case of quantitative detection, an internal standard is incorporated in the multiplex PCR by including a known amount of standard DNA target and an associated primer pair in the reaction. The quantity of amplicons produced from the internal standard target is then correlated to the quantity of the amplicons produced the target DNA in the sample in order to determine the amount of target DNA in the initial sample.

The detection of amplicons indicates the presence of target nucleic acid in the sample. When gel electrophoresis is used, amplicons are confirmed by their size, as

predicted by the location in the respective target sequences corresponding to the amplification primers used in the PCR.

A specific embodiment of the present invention provides a method in which the amplicons are detected using agarose gel electrophoresis with staining, for example, using ethidium bromide or SyBr Green™.

Alternatively, the amplicons are detected using a hybridization assay. In this case, nucleic acid probes that are complementary to regions of the amplified DNA are hybridized to the denatured amplicons. The probes are labelled to allow visualisation, and, optionally, quantification of the amplicons hybridized.

The term "probe", as used herein, refers to a nucleic acid oligomer that hybridizes specifically to a target sequence in a nucleic acid, which, in the context of the present invention, is an amplicon, under standard conditions that promote hybridization. This allows detection of the amplicon. Detection may either be direct (i.e., resulting from a probe hybridizing directly to the amplicon sequence) or indirect (i.e., resulting from a probe hybridizing to an intermediate molecular structure that links the probe to the target amplicon). A probe's "target" generally refers to a sequence within (i.e., a subset of) an amplified nucleic acid sequence which hybridizes specifically to at least a portion of a probe oligomer using standard hydrogen bonding (i.e., base pairing). A probe may comprise target-specific sequences and other sequences that contribute to three-dimensional conformation of the probe (e.g., as described in Lizardi et al., U.S. Pat. Nos. 5,118,801 and 5,312,728). Sequences that are "sufficiently complementary" allow stable hybridization of a probe oligomer to a target sequence in the amplicon even thought it is not completely complementary to the probe's target-specific sequence.

By "sufficiently complementary" is meant a contiguous nucleic acid base sequence that is capable of hybridizing to another base sequence by hydrogen bonding between a series of complementary bases. Complementary base sequences may be complementary at each position in the base sequence of an oligomer using standard base pairing or may contain one or more residues that are not complementary using standard hydrogen bonding (including a basic "nucleotides"), but in which the entire complementary base sequence is capable of specifically hybridizing with another base sequence in appropriate hybridization conditions. Contiguous bases are preferably at

least about 80%, more preferably at least about 90%, and most preferably greater than 95% complementary to a sequence to which an oligomer is intended to specifically hybridize. To those skilled in the art, appropriate hybridization conditions are well known, can be predicted based on base composition, or can be determined empirically by using routine testing (e.g., see Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) at §1.90-1.91, 7.37-7.57, 9.47-9.51 and 11.47-11.57 particularly at §9.50-9.51, 11.12-11.13, 11.45-11.47 and 11.55-11.57).

The terms "label" and "detectable label", as used herein, refer to a molecular moiety or compound that can be detected or can lead to a detectable response. A label is joined, directly or indirectly, to a nucleic acid probe. Direct labelling can occur through bonds or interactions that link the label to the probe, including covalent bonds or non-covalent interactions (e.g., hydrogen bonding, hydrophobic and ionic interactions) or through formation of chelates or co-ordination complexes. Indirect labelling can occur through use of a bridging moiety or "linker," such as an antibody or additional oligonucleotide(s), which is either directly or indirectly labelled, and which can amplify a detectable signal. A label can be any known detectable moiety, such as, for example, a radionuclide, ligand (e.g., biotin, avidin), enzyme or enzyme substrate, reactive group, chromophore, such as a dye or particle that imparts a detectable colour (e.g., latex or metal particles), luminescent compound (e.g., bioluminescent, phosphorescent or chemiluminescent labels) and fluorescent compound.

Preferably, the label on a labelled probe is detectable in a homogeneous assay system, i.e., where, in a mixture, bound labelled probe exhibits a detectable change, such as stability or differential degradation, compared to unbound labelled probe, without physically removing hybridized from non-hybridized forms of the label or labelled probe. A "homogeneous detectable label" refers to a label whose presence can be detected in a homogeneous fashion, for example, as previously described in detail in Arnold et al., U.S. Pat. No. 5,283,174; Woodhead et al., U.S. Pat. No. 5,656,207; and Nelson et al., U.S. Pat. No. 5,658,737. Examples of labels that can be used in a homogenous hybridization assay include, but are not limited to, chemiluminescent compounds (e.g., see U.S. Patent Nos. 5,656,207, 5,658,737 and 5,639,604), such as acridinium ester ("AE") compounds, including standard AE or derivatives thereof.

Synthesis and methods of attaching labels to nucleic acids and detecting labels are well known in the art (e.g., see Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd ed. (Cold Spring Harbor Laboratory Press, Cold Spring Habor, NY, 1989), Chapter 10; U.S. Patent Nos. 5,658,737, 5,656,207, 5,547,842, 5,283,174, 4,581,333 and European Patent Application No. 0 747 706).

In accordance with another embodiment of the present invention, where the amplicons are detected using an assay without prior separation of the amplicons, they are detected using different detectable molecules to allow the amplicons from the different primer pairs to be distinguishable. For example, probes used to hybridize to the various amplicons can be labelled with labels that are detectable at different wavelengths.

In accordance with another embodiment of the present invention, the amplicon production is monitored in real-time using procedures known in the art (e.g. see U.S. Patent No. 6, 569,627).

<u>Application of Universal Multiplex PCR</u>

The method of the present invention can be readily incorporated into existing PCR products, or used to design a new generation of screening tests. In this manner a diagnostic screening assay of the present invention can be easily performed by a clinician and results rapidly obtained. It is fully contemplated that the present invention includes a kit providing the materials for performing the universal multiplex PCR method described herein, which materials are provided in a suitable container or plurality of containers. The kit is optionally provided with instructions for use.

The method of the present invention has particular application in the diagnosis of infectious diseases where many target organisms can be simultaneously screened in a timely and affordable fashion. In a specific embodiment of the present invention one or more of the target organisms is a virus, which may be a retrovirus, a bacteria, a fungus or a parasite. As would be readily appreciated by a worker skilled in the art, the present invention has the potential to be applied to many other areas of DNA-based and RNA-based diagnostics, such as cancer diagnosis using detection of cancer markers, forensics, genetic testing and profiling, etc. Such diagnostic tests can be applied to RNA and DNA samples obtained from any organism, such as animals and humans.

Reverse transcriptase-mediated PCR (RT-PCR) assays are derivatives of PCR in which amplification is preceded by reverse transcription of mRNA into cDNA. Accordingly, one embodiment of the present invention provides a multiplex RT-PCR method comprising the step of reverse transcribing target RNA into cDNA followed by amplification of the cDNA using the multiplex PCR described herein. Such a multiplex RT-PCR method may be used to detect any target RNA such as, but not limited to viral RNA. An additional application of the multiplex RT-PCR method of the present invention is in the area of gene expression analysis. In this case the target RNA is mRNA.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLES

Example 1: Multiplex PCR of Ten MRSA Gene Targets

Primer design

Primers were designed to give amplicons that ranged in size between 200 to 900 bp. Where detection was by agarose gel electrophoresis, a size difference of at least 20 bp between individual amplicons was preferred. Detection by a more sensitive technique like capillary gel electrophoresis can be employed in accordance with the present invention to resolve a size difference of as little as 1 bp.

When designing primers, the structural properties of each primer set were selected according to the following primer selection criteria:

Melting Temperature (T_m)	55 – 57°C
%GC	40 – 50%
optimal primer length	22 nt
Primer size (range)	18 – 27 nt

The above conditions were specified in the primer-design program Primer3 (freely available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). After generating a candidate design, the new primer was checked for internal stability and mispriming according to methods well known in the art.

In cases where a primer pair did not work immediately, a new primer pair was designed according to the same principles, after reassessing the genomic structure and/or stability at that priming site. The reaction conditions were not adjusted in these cases.

Reagent	**Working Concentration**	**Volume (µl)/ Reaction**	**Final concentration**
AmpliTaq Gold® PCR Buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3)	10 ×	3	1 ×
Mg_2Cl	25 mM	9	7.5 mM
dNTPs	1.25 (mM) each	6	0.25 mM
DNA template	variable	3	variable
AmpliTaq Gold® enzyme	5U/µl	0.5	2.5 U/30.5 µl
Primer Master Mix (primers + TE Buffer: 10mM Tris-HCl, pH 8.0 and 1mM EDTA)	0.05 µM each	6	0.01 µM each
H_2O	--	3	--
TOTAL VOLUME = 30.5			

AmpliTaq Gold®, which is a hot start DNA polymerase enzyme, was used in the PCR steps. In this case, reactants were not wasted in the formation of unintended products and increased yields of the specific products were achieved. The final concentration of magnesium chloride in the reaction mix was 7.5 mM. Equal volumes of each primer were added together to prepare the master mix containing all of the primers to be used in the multiplex assay. The concentration of each primer in this Primer Master Mix was 0.05 µM. As a result, the final primer concentration in the PCR reaction mixture was approximately 0.01 µM.

It is contemplated that fractions or multiples of these values can be used in accordance with the present invention if reagent volume ratios are preserved.

PCR thermal cycler program

Step 1: Initial Denaturation

Time (min)	Temperature (°C)
10:00	95

Step 2: 20 cycles of Touchdown PCR

Time (min)	Temperature (°C)	Touchdown
0:20	95	none
1:00	63	decrease by 0.5°C each cycle
1:00	72	none

Touchdown or step-down PCR refers to the incremental decrease of the annealing temperature with each cycle. Here, the annealing temperature was decreased by 0.5°C in each cycle. The objective was to increase the efficiency of each successive amplification step, while maintaining more rigorous primer specificity in the initial amplification steps.

Step 3: 25 cycles of regular PCR

Time (min)	Temperature (°C)
0:20	95
0:45	56
1:00	72

Step 4: Final elongation

Time (min)	Temperature (°C)
7:00	72
HOLD	6

Primer pairs used in this example are herein provided as SEQ ID Nos. 1 – 20. A GeneAmp™ PCR System 9600 thermal cycler was programmed as detailed above. PCR

products were detected by agarose gel electrophoresis, followed by staining with ethidium bromide. PCR products were identified based on size comparison to a standard DNA ladder. Figure 2 shows the results of this experiment.

Ten genes expressed in methicillin-resistant Staphylococcus aureus (MRSA) were identified from GenBank, as outlined below. Detection of 10 genetic targets, corresponding to these ten different genes in methicillin-resistant Staphylococcus aureus was achieved using agarose gel electrophoresis and staining as described above. As illustrated in Figure 2 (Lane 1), from top to bottom, the ten genes are: (1) agr, 823 bp (ACCESSION M21854)(2) clumping factor, 726 bp (ACCESSION Z18852); (3) 16S rRNA, 653 bp (ACCESSION X68417); (4) hld, 554 bp (ACCESSION X17301); (5) femA, 419 bp (ACCESSION X17688 M23918); (6) rho, 376 bp (ACCESSION AF333962) (7) DNA polymerase III, 314 bp (ACCESSION Z48003 L39156); (8) nuclease, 282 bp (ACCESSION V01281 J01785 M10924); (9) 23S rRNA, 244 bp, (ACCESSION X68425); and (10) hsp60, 212 bp (ACCESSION AF060189).

Example 2: Multplex Assay Using Bacterial Extracts

Method

A 0.5 McFarland standard (1 x 10^8 CFU/ml) of MRSA bacteria was made up in sterile saline. Serial dilutions were made using sterile saline. The bacteria from 100 µl of each dilution were pelleted and resuspended in Lysis Buffer (50mM Tris-HCl (pH 8.0), 50mM NaCl, and 5 mM EDTA (pH 8.0) for DNA extraction. Colony counts of bacteria on agar plates corroborated the accuracy of the McFarland standard. 2 µl from each DNA sample (100 µl each) was used for the method of the present invention as described in Example 1.

Results

The sensitivity of the method of the present invention is illustrated in Figure 3, with 10 amplicons visible in Lanes 1 and 2. Lanes 3 to 5 show progressive loss of amplified targets with a decrease in the initial concentration (CFU/ml) of bacteria. A theoretical detection limit of approximately 500 CFU/ml (2 µl/PCR reaction × 500 CFU/1000 µl = 1 CFU / PCR reaction) was indicated in this example.

Example 3: Magnesium Concentration

Narrow Range

The effect of varying magnesium chloride concentrations was investigated in accordance with an embodiment of the present invention. A series of multiplex PCRs were performed according to the method set out in Example 1, except that various final concentrations of $MgCl_2$ were employed. As depicted in Figure 4, final $MgCl_2$ concentrations of 5.0 mM to 10.0 mM allowed detection of the 10 target genes by agarose gel electrophoresis (Fig. 4 – Lanes 2, 3 and 4). No amplicons were detected when a final concentration of 2.5 mM $MgCl_2$ was provided in the PCR reaction mixture (Lane 1). Only 9 amplicons were visible (Lane 5) when the final concentration of $MgCl_2$ was increased to 12.5mM.

Broad Range

The effect of varying Mg^{2+} was further investigated by performing a second series of multiplex PCRs, using various Mg^{2+} concentrations, under the following conditions:

Primer concentration (final)	0.02 μM each
PCR program	40 cycles: 95°C denaturation for 20 seconds, 56°C annealing for 1 minute and 72°C extension for 1 minute
dNTPs	0.25 mM
DNA polymerase enzyme	Native Taq, 2.5 U/ 30 μl reaction
PCR Buffer	50 mM KCl, 10 mM Tris-HCl

A 10plex PCR reaction was performed using $MgCl_2$ concentrations varying from 1.5-15 mM. Following PCR the products of the reaction were separated by agarose gel electrophoresis and visualised using ethidium bromide. The lanes of the agarose gel contained samples from multiplex PCRs having the following $MgCl_2$ concentrations (Figure 5):

Lane #	Final concentration of $MgCl_2$ (mM)
1	100 bp ladder
2	1.5
3	2.5
4	5.0
5	6.0
6	7.5
7	10
8	12.5
9	15
10	100 bp ladder

The primers used in this example were directed towards genes present in methicillin-resistant *Staphylococcus aureus* (ATCC Strain 43300). DNA for the PCR reactions was obtained from pure bacterial colonies using a boiling lysis protocol.

Gene name	Primer name	Primer sequences (5'-3')	SEQ ID	Amplicon size (bp)
rho	rho-3 rho-4	aggtcaacgtggtttaatagtg ccatctggaacagagttatttg	21 22	877
agr	agr-1 agr-2	gccataaggatgtgaatgtatg cagctatacagtgcatttgcta	7 8	823
DNA polymerase	DNApol-3 DNApol-4	ggcaatgacagagcaaca tatggcgaccacttttaagttc	23 24	776
16S rRNA	16S-1 16S-2	ggattagataccctggtagtcc cttcgggtgttacaaactctc	11 12	653
23S rRNA	23S-3 23S-4	ctaacgacgatatgctttgg tttactgcttaaccttgcatca	25 26	599
DNA polymerase	DNApol-5 DNApol-6	cttactttacaattcgtgcgtcag tatttctttgactacgccatacgc	27 28	495
femA	femA-1 femA-2	taccgctttaaacgtggatt gatatcacacacttgcaaacac	15 16	419
DNA polymerase	DNApol-1 DNApol-2	gtagaattaacgcaacatcacc cacgctgtacctaccaataatc	19 20	314
lipase	lip-1 lip-2	ggtcataacaatatggttgcat cattatcgtctgatgtccaaat	29 30	253
helicase	hel-1 hel-2	cgagatttagaccaaatgacag atcatacgtgtggctaactgat	31 32	188

Final $MgCl_2$ concentrations of 5.0 mM to 12.5 mM were shown to allow detection of 10 target genes by agarose gel electrophoresis (Figure 5, Lanes #4-8). Only

one amplicon was detected when the $MgCl_2$ concentration was 1.5 mM (Figure 5, Lane #1).

Example 4: Primer Concentration

The effect of varying primer concentrations was further investigated by performing a second series of multiplex PCRs using the following PCR conditions:

$MgCl_2$ concentration	7.5 mM
PCR program	20 cycles: 95°C denaturation for 20 seconds, touchdown PCR with annealing temperature decreasing by 0.5°C each cycle from 63°C to 56°C for 1 minute, 72°c extension for 1 minute; plus 25 cycles: 95°C denaturation for 20 seconds, 56°c annealing for 1 minute and 72°C extension for 1 minute
dNTPs	0.25 mM
DNA polymerase enzyme	AmpliTaq Gold®, 2.5 U/30 µl reaction
PCR Buffer	50 mM KCl 10 mM Tris-HCl

10plex PCRs were performed using primer concentrations varying from 0.0025 – 0.3 uM. Following PCR the products of the reaction were separated by agarose gel electrophoresis and visualised using ethidium bromide. The agarose gel shown in Figure 6 demonstrates that high magnesium is the most important factor for universal multiplex PCR. Even at high primer concentrations, it was possible to clearly see at least a 6plex. The lanes of the agarose gel contained samples from multiplex PCRs having the following primer concentrations:

Lane #	Final primer concentration (µM)
1	100 bp ladder
2	0.3
3	0.2
4	0.1
5	0.05
6	0.02

Lane #	Final primer concentration (μM)
7	0.01
8	0.005
9	0.0025
10	100 bp ladder

The primers and genetic targets employed were the same as those used in Example 3 (Broad range).

Typical PCR reactions use a final primer concentration of 1 μM each. In accordance with the present invention, however, a final primer concentration of between 0.005-0.02 μM is optimal (Lanes #6-8). At higher primer concentrations, the larger amplicons get blurred and some of the amplicons are not amplified. At lower concentrations, the amplicons are only faintly visible following agarose gel electrophoresis. The amplicons are probably present, but a more sensitive detection system is required.

The benefits resulting from a reduced primer concentration are: (i) no "dropping" of amplicons because more efficiently amplified primers are present in limiting amounts so that they do not use up all of the reagents even if they are preferentially amplified; and (ii) similar amounts of amplicon product are produced for each primer pair as evidenced by the similar intensities on the agarose gel.

Example 5: Additional primer pairs

One of the surprising advantages of the present invention is that primers can be added or subtracted from a multiplex panel without altering the reaction concentrations or conditions. This is an advantage because it eliminates tedious and time-consuming empirical optimisation, which is one of the major constraints of standard multiplex PCR.

The effect of adding and subtracting different primer pairs was investigated in using multiplex PCR according to one embodiment of the present invention. The multiplex PCR parameters were as described in Example 3 (Broad range) except that

AmpliTaq Gold® was used in place of Native Taq and the final $MgCl_2$ concentration was 7.5 mM for all PCRs.

Following PCR the products of the reaction were separated by agarose gel electrophoresis and visualised using ethidium bromide. The agarose gel shown in Figure 7 demonstrates that the same reaction conditions are suitable for a 2plex, a 5plex and a 7plex. In the case of the 5plex, the reaction mixture was identical to that of the 2plex except that three additional primer pairs were added. Similarly, the 7plex reaction mixture was identical to that of the 5plex except that two additional primer pairs were added. The lanes of the agarose gel contained samples from multiplex PCRs as follows:

Lane #	Multiplex primers
1	100 bp marker ladder
2	2plex: lip-1/2, hel-1/2
3	5plex: lip-1/2, hel-1/2, DNA pol-1/2, femA-1/2, DNA pol-5/6
4	7plex: lip-1/2, hel-1/2, DNA pol-1/2, femA-1/2, DNA pol-5/6, 23S-3/4, 16S-1/2

__Example 6: Primer Melting Temperatures__

The effect of using primers having varying melting temperatures (Tm's) was investigated in accordance with an embodiment of the present invention. Tm's were calculated using the "nearest neighbour" method referenced in U.S. Patent No. 5,582,989. An 8plex reaction was performed. There was a difference of 19°C between the highest and lowest primer Tm's (65°C and 46°C) in this example. Within individual primer pairs, the greatest difference in Tm was 13°C for the *Mycoplasma hominis* primers.

The multiplex PCRs were performed as described in Example 4 except that the primer concentrations were maintained at 0.02 µM each.

After the PCR, agarose gel electrophoresis was performed. The gel from this investigation is shown in Figure 8. The lanes of the agarose gel contained samples from multiplex PCRs as follows:

Lane #	
1	100 bp ladder
2	8plex

The primers and genetic targets used in this Example were as follows:

Organism	Primer name	Gene name	Amplicon (bp)	Sequence 5'-3' (SEQ ID NO)	Tm (°C)
Mycoplasma genitalium	MGS-1 MGS-2	Adhesin gene	673	gagcctttctaaccgctgc (33) gtggggttgaaggatgattg (34)	52 52
Human papillomavirus	FK-16-3 FK-16-4	HPV 16	499	gtcaaaagccactgtgtcct (35) ccatccattacatcccgtac (36)	49 49
Ureaplasma urealyticum	U4 U5	Urease	429	caatctgctcgtgaagtattac (37) acgacgtccataagcaact (38)	46 47
Neisseria gonorrhoeae	HO1 HO3	cppB	390	gctacgcatacccgcgttgc (39) cgaagaccttcgagcagaca (40)	60 53
Mycoplasma hominis	MYCOH1 MYCOH2	16S rRNA	334	caatggctaatgccggatacgc (41) ggtaccgtcagtctgcaat (42)	60 47
Chlamydia trachomatis	KL1 KL2	Plasmid	241	tccggagcgagttacgaaga (43) aatcaatgcccgggattggt (44)	55 58
Human papillomavirus	Soler-18	HPV 18	201	cgacaggaacgactccaacga (45) gctggtaaatgttgatgattaact (46)	57 49
Human papillomavirus	FK-31-1 FK-31-2	HPV 31	153	ctacagtaagcattgtgctatgc (47) acgtaatggagaggttgcaataaccc (48)	49 59

It can be seen that successful multiplex PCR can be performed using primers where the Tm varies significantly between the highest and lowest values. This is advantageous because it broadens the types of primers that can be used in the multiplex PCR method of the present invention, in comparison to the types that may be used in standard multiplex PCRs.

Example 7: DNA Polymerase Enzymes

The effect of different DNA polymerase enzymes on multiplex PCR was investigated in accordance with one embodiment of the present invention. A series of 10plex PCRs were performed with various DNA polymerase enzymes.

The experimental parameters were the same as in Example 4 except that the final primer concentration was 0.02 μM each and the type of DNA polymerase enzyme varied as indicated. Each 30 μl PCR reaction used 2.5 units of the particular enzyme. The primers and genetic targets were the same as listed in Example 3 (broad range).

The PCR reaction buffer contained final concentrations of 50 mM KCl and 10 mM Tris-HCl. No other cations, such as NH_4^+, or additives were required. In separate experiments not shown here, it was found that multiplex PCR with AmpliTaq Gold® can work be performed in sterile water in the absence of any buffer, however, this finding does not seem to be general to all enzymes.

After the PCR, agarose gel electrophoresis was performed. The gel from this investigation is shown in Figure 9. The lanes of the agarose gel contained samples from multiplex PCRs as follows:

Lane #	DNA polymerase enzyme
1	100 bp ladder
2	AmpliTaq Gold® (Applied Biosystems)
3	Recombinant Taq (Roche)
4	PfuTurbo® Hotstart (Stratagene)
5	HotStarTaq® (Qiagen)
6	100 bp ladder

This investigation demonstrated that successful multiplex PCR can be performed according to the present invention using either hotstart or non-hotstart enzymes (i.e. with or without a hot start).

Example 8: Amount of enzyme

The effect of the amount of enzyme on the multiplex PCR was investigated by performing a series of PCRs that were identical except that the amount of enzyme, recombinant Taq polymerase, was varied.

In this investigation, six 10plex PCR reactions were performed according to the present invention, using reaction conditions as defined in Example 4 except that recombinant Taq polymerase in place of AmpliTaq Gold®, the amount of enzyme was

varied between reactions and the final primer concentration was 0.02 μM each. The primers and genetic targets were the same as those used in Example 3 (broad range).

Following PCR the products of the reactions were separated by agarose gel electrophoresis and visualised using ethidium bromide. The lanes of the agarose gel contained samples from multiplex PCRs as follows:

Lane #	Enzyme Units / 30 μL reaction
1	100 bp ladder
2	0.5
3	1.0
4	1.25
5	1.5
6	2.0
7	2.5
8	100 bp ladder

The agarose gel shown in Figure 10 demonstrates that multiplex PCR according to the present invention can be performed successfully with as little as 0.5 U of enzyme per 30 μl reaction. A conventional PCR requires 1 – 2 U of enzyme per 30 μl reaction.

Generally, the cost of the enzyme is the most expensive component of a PCR reaction. Therefore, it is advantageous to use less enzyme in a multiplex PCR reaction. This is another advantage of this invention compared to the multiplex PCR described U.S. Patent No. 5,582,989, which uses increased amounts of enzyme.

Example 9: Fast Reaction

The effect of reducing reaction times was investigated in accordance with one embodiment of the present invention. A 10plex PCR reaction was performed for 40 cycles with 95°C denaturation for 1 second, 56°C annealing for 5 seconds, and 72°C extension for 5 seconds. The PCR components were as follows:

Primer concentration (final)	0.02 μM each
dNTPs	0.25 mM
DNA polymerase enzyme	Recombinant Taq, 5 U/ 30 μl reaction

PCR Buffer	50 mM KCl, 10 mM Tris-HCl

The primers and genetic targets were the same as those used in Example 3 (broad range). Following PCR the products of the reactions were separated by agarose gel electrophoresis and visualised using ethidium bromide. The lanes of the agarose gel contained samples from multiplex PCRs as follows:

Lane #	Sample
1	100 bp ladder
2	10plex with 95°C denaturation for 1 second, 56°C annealing for 5 seconds, and 72°C extension for 5 seconds

The agarose gel picture shown in Figure 11 demonstrates that a 10plex reaction can be performed with very fast reaction times. This is an advantage over standard multiplex PCR because results can be obtained much faster.

Example 10: Two temperature PCR

A two-temperature multiplex PCR according to the present invention was performed in which the annealing and extension steps were performed at a single temperature. A 10plex PCR was performed for 40 cycles with 95°C denaturation for 1 second, and annealing/extension at 56°C for 10 seconds. The components of the PCR were as described in Example 9, including the primers.

Following PCR the products of the reactions were separated by agarose gel electrophoresis and visualised using ethidium bromide. The lanes of the agarose gel contained samples from multiplex PCRs as follows:

Lane #	Reaction time
1	100 bp ladder
2	10plex with 95°C denaturation for 1 second, 56°C annealing/extension for 10 seconds

The picture of the agarose gel in Figure 12 demonstrates that a 10plex reaction can be performed using a two-temperature PCR program.

Example 11: dNTP Concentration

The effect of dNTP concentration in the multiplex PCR of the present invention was investigated by performing a series of 10plex reactions in which all the conditions were identical except the amount of dNTPs, which was varied from 0.083 – 1.33 mM. The PCR conditions were the same as described in Example 4 except that the final primer concentration was 0.02 μM and the dNTP concentrations were varied. The primers and genetic targets were as defined in Example 3 (broad range).

Following PCR the products of the reaction were separated by agarose gel electrophoresis and visualised using ethidium bromide. The lanes of the agarose gel contained samples from multiplex PCRs as follows:

Lane #	Final dNTP concentration (mM)
1	Ladder
2	0.083
3	0.167
4	0.25
5	0.33
6	0.67
7	1.33
8	Ladder

The agarose gel depicted in Figure 13 demonstrates that the universal multiplex PCR of the present invention can be successfully performed using a wide range of dNTP concentrations, and likely outside the range shown here. These findings are in contrast to what has been taught in the art, for example U.S. Patent No. 5,582,989.

Example 12: Number of Targets

In this investigation, a multiplex PCR reaction according to the present invention was performed to amplify 16 target sequences. The PCR conditions were as described in

Example 4 except that the final primer concentration was 0.02 μM each and the 20 cycles of touchdown PCR started at 70°C and ended at 60°C.

The primers used in this example were directed toward various exons in the human CFTR gene. Template DNA for the PCRs was obtained from whole human blood using a standard DNA extraction protocol.

Primer name	Primer sequences (5'-3')	SEQ ID NO	Amplicon size (bp)
CF1	F- gactttaaagctgtcaagccgtgtt R- aattactattatctgacccaggaaaactcc	49 50	185
CF2	F- ttttgcagagaatgggatagagagc R- cacctattcaccagatttcgtagtcttttc	51 52	208
CF3	F- gatgtgaatttagatgtgggcatgg R- tgcttgggagaaatgaaacaaagtg	53 54	231
CF4	F- caaagatgctgatttgtatttattagactctcc R- taatcttgaatcctggcccagtagg	55 56	257
CF5	F- acagtggaagaatttcattctgttctca R- ccattcacagtagcttacccatagagg	57 58	270
CF6	F- gtgatatatgattacattagaaggaagatgtgc R- tttttcaattccagaaacagaatataaagca	59 60	298
CF7	F- gaacttgatggtaagtacatgggtgtttc R- tgcacacaaagtgtgtagaatgatgtc	61 62	322
CF8	F- tccaaagatatagcaattttggatgacc R- attttgggaaagatggtcctttgtg	63 64	347
CF9	F- ggtcatatgatgtggagccaggtta R- gctccaagagagtcataccatgtttgt	65 66	368
CF10	F- aaagcccgacaaataaccaagtgac R- agcagtgttcaaatctcaccctctg	67 68	399
CF11	F- ggccatgtgcttttcaaactaattg R- acaagacactacacccatacattctcct	69 70	436
CF12	F- tccaattccttatggccagtttctc R- tttgcagagtaatatgaatttcttgagtacaa	71 72	484
CF13	F- gaacacataaaagattcaattataatcaccttg R- tggtttccttatatcattgaactgctg	73 74	515
CF14	F- ctcattttaagtctcctctaaagatgaaaagtc R- acaacagaggcagtttacagaagatactca	75 76	550
CF15	F- agcagctattttttatgggacattttcag R- tcacttatttccaagccagtttcttga	77 78	599
CF16b	F- caagcttaaaaggactatggacacttcg R- tgttaaaatggaaatgaaggtaacagca	79 80	194

Following PCR the products of the reaction were separated by gel electrophoresis with low-melting temperature agarose (MetaPhor™ agarose, Cambrex Bio Science Rockland Inc.) and visualised using ethidium bromide. The agarose gel depicted in Figure 14 shows the results of this investigation, which demonstrated simultaneous amplification of 16 target sequences. The lanes of the agarose gel contained samples from multiplex PCRs as follows:

Lane #	Multiplex PCR reaction
1	50 bp ladder
2	16plex

Example 13: Stability

For reaction mixtures to be supplied in a kit it is useful for them to exhibit good stability under standard storage conditions. In order to investigate reaction mixture stability, mixtures were prepared that contained enzyme or did not contain enzyme. The mixtures were stored at room temperature, 4°C or -20°C for eight weeks and then used in multiplex PCR. In this investigation, the multiplex PCR reactions were performed using the same conditions as described in Example 4, except that the final primer concentration was 0.01 μM each. The primers and genetic targets were the same as defined in Example 3 (broad range).

Following PCR the products of the reaction were separated by agarose gel electrophoresis and visualised using ethidium bromide. The agarose gel depicted in Figure 15 shows the results of this investigation. The lanes of the agarose gel contained samples from multiplex PCRs as follows:

Lane #	Storage temperature (°C)	Enzyme premixed?
1	100 bp ladder	--
2	-20	Yes
3	4	Yes
4	RT	Yes
5	-20	No

Lane #	Storage temperature (°C)	Enzyme premixed?
6	4	No
7	RT	No
8	100 bp ladder	--

This study demonstrated that reaction mixtures containing primers, buffer, magnesium, and dNTPs are stable at room temperature, 4°C, and -20°C for at least 8 weeks. This was found for mixtures in which the enzyme was added after storage and for mixtures in which the enzyme was premixed with the reaction mixture prior to storage. Therefore, a specific embodiment of the present invention provides universal multiplex PCR master mixtures (plus or minus enzyme) that can be shipped and stored at room temperature, 4°C or -20°C. The current standard of practice is to store and ship enzyme and master mixes at 4°C or -20°C only.

<u>Example 14: Detection Using Capillary Electrophoresis</u>

In this investigation, a multiplex PCR reaction was performed to amplify 5 target sequences. The multiplex PCR reaction was performed using the same conditions as described in Example 4, except that the final primer concentration was 0.02 μM each. The primers and genetic targets were lip-1/2, femA-1/2, 23S-3/4, agr-1/2 and DNA pol-1/2, as defined in Example 3.

Following PCR, agarose gel electrophoresis was performed with ethidium bromide staining. The resulting gel is depicted in Figure 16A, in which the lanes of the agarose gel contained samples from multiplex PCRs as follows:

Lane #	Multiplex reaction
1	50 bp ladder
2	5plex

In addition, the products of the reaction were separated by capillary gel electrophoresis using an ABI 3100 capillary electrophoresis machine (Applied Biosystems). Primers were labelled with 6-FAM fluorescent dye. The computer display

readout depicted in Figure 16B shows the results of this investigation. The labelled peaks correspond to the fluorescent detection of the 5plex amplicons.

Example 15: Amplicon size Using AmpliTaq Gold®

According to the manufacturer, AmpliTaq Gold® can efficiently amplify templates up to 5 kb long, but 100-1000 bases are more typical and easier to amplify. This was found to be true for the use of AmpliTaq Gold® in the universal PCR method of the present invention.

In this investigation, two multiplex PCR reactions were performed according to the present invention, using the same reaction conditions as Example 4 except that the final primer concentration was 0.02 µM. Both of the 4plex reactions used the rho-3/4, agr-1/2, and DNA pol-3/4 primers described in Example 3 (broad range). The first 4plex added the additional primer pair named 16S rRNA-5/6 and the second 4plex added an additional primer pair named 16S rRNA-7/8.

The additional primers had the following sequences:

Name	Primer sequence (5'-3')	SEQ ID NO:	Amplicon size (bp)
16S rRNA-5 16S rRNA-6	ttttatggagagtttgatcctg agctcctaaaaggttactccac	41 42	1470
16S rRNA-7 16S rRNA-8	gtgcctaatacatgcaagtcg caccttccgatacggctac	43 44	1490

Following PCR the products of the reaction were separated by agarose gel electrophoresis and visualised using ethidium bromide. The agarose gel depicted in Figure 17 demonstrates successful multiplex PCR when the reaction included amplification of amplicons up to 1490 bp in length. Following agarose gel electrophoresis and ethidium bromide staining, the larger amplicons were observed as fainter bands than those of the smaller ones. The lanes of the agarose gel contained samples from multiplex PCRs as follows:

Lane #	Multiplex reaction
1	100 bp ladder

2	4plex, largest amplicon 1470 bp
3	4plex, largest amplicon 1490 bp
4	100 bp ladder

Example 16: Comparison to Standard Multiplex PCR

In the past, multiplex PCR has been optimized through the use of conditions that are different from non-multiplex PCR, such as: an increased enzyme concentration; an extended reaction time (significantly increased cycle duration); an increased dNTP concentration; and carefully balanced primer compositions (well-matched primer pairs having similar melting temperatures).

In contrast, however, it has now been found that these four conditions are not necessary for effective amplification using the universal multiplex PCR of the present invention. First, enzyme concentration can be as low as 0.5 units per 30 µl reaction, which is not different from the concentration used in standard PCRs. Second, no extended reaction time is necessary for performing universal multiplex PCR. In fact, these reactions can be performed using a denaturing time of 1 second, annealing time of 30 seconds and an extension time 30 seconds. Third, dNTP concentration in universal multiplex PCR can be as low as 80 µM, which is not increased in comparison to standard PCR. Fourth, it is not necessary to design the primers for use in the universal multiplex PCR to have melting temperatures within a narrow range. In fact, successful amplification is achieved using the method of the present invention when the melting temperatures of the primers are within a 26°C range (calculated using the "nearest neighbour" method). Within individual primer pairs the melting temperatures can vary dramatically, for example, by as much as 13°C.

In order to compare the multiplex reaction of the present invention with that of a standard multiplex reaction, a 10plex reaction was performed using the conditions summarized in Example 3 (broad range) and a second 10plex reaction was performed using the conditions described in Chamberlain et al. (1988) *Nucleic Acids Res.*, 16(23):11141-56. In each case the primers and genetic targets were the same as those defined in Example 3 (broad range).

Specifically, standard multiplex PCR was performed using the following conditions:

Reagent	Final concentration
Tris-HCl (ph 8.3)	67 mM
Magnesium chloride	6.7 mM
Ammonium sulphate	16.6 mM
EDTA	6.7 uM
BSA	170 µg/ml
dNTPs	1.5 mM
Recombinant Taq	100 units/ml
Primers	1 uM each
Final reaction volume = 100 µl	

The Standard PCR was performed as follows:

- 94°C for 7 minutes
- 25 cycles of 94°C for 1 minute, 55°C for 45 seconds, 65°C for 3.5 minutes
- 65°C for 7 minutes

Following PCR the products of the reaction were separated by agarose gel electrophoresis and visualised using ethidium bromide. The agarose gel depicted in Figure 18 shows the results of this investigation. The lanes of the agarose gel contained samples from multiplex PCRs as follows:

Lane #	Multiplex PCR reaction
1	100 bp ladder
2	10plex (Standard)
3	10plex (universal multiplex PCR)
4	100 bp ladder

The standard multiplex PCR method was found to allow multiplex PCR to occur, but amplification efficiency was uneven. In contrast, consistent amplification efficiency was observed using the universal multiplex PCR of the present invention.

Conclusions

The present invention provides a single set of multiplex PCR conditions that will work with primers designed according to any primer design protocol. By employing this single set of multiplex PCR conditions, primer pairs designed in accordance with the primer design protocol of the present invention can be added or removed without having to change the reaction conditions of the enhanced multiplex PCR method.

The present invention amplifies all of the genetic targets if they are present. At least 16 genetic targets can be amplified simultaneously, according to one embodiment of the present invention. More efficiently amplified primers do not overwhelm less efficiently amplified primers in the reaction mix. A particular advantage of the present invention is that amplification is efficient enough to produce PCR products or amplicons that can be detected with inexpensive and simple methods like agarose gel electrophoresis with ethidium bromide staining.

In effect, the present invention creates a common platform that researchers and clinicians can use to develop new multiplex PCR tests. As new genetic targets are discovered, the method of the present invention can be adapted to include primers for one or more of these targets in the reaction mixture, for amplification with the existing targets, without requiring any changes to reaction conditions. Conversely, if a certain genetic target is no longer desirable, its primer pair can be removed from the existing assay, without requiring any changes to the reaction conditions.

To summarize, the present invention includes the advantages of (1) significant savings in reagent costs and technologist time and labour due to the elimination of optimization procedures; (2) rapid product development: different PCR primer pairs will work together right away without competitive inhibition; and (3) flexibility: new PCR primers can be added or subtracted from existing assays without adjusting reaction conditions.

By selecting target-specific primers, the present invention can be used to develop diagnostic assays in a variety of fields. For example, the present invention can be used to provide high-throughput, sensitive and specific diagnostic tests for infectious diseases, screening panels for genetic diseases, or for the detection of disease-causing genes. In

particular, the present invention can be tailored to provide a screening method and kit to detect potential causative organisms of human encephalitis in cerebrospinal (CSF) samples (e.g. herpes simplex virus, human herpes virus 6, *Cryptococcus, Listeria, H. influenzae* type B, S. pneumoniae, E. coli, etc.) The present invention also finds application in the fields of blood product screening and genetic testing such as prenatal screening for genetic diseases and detection of cancer-causing genes or cancer markers in pathology samples, for example.

In the case of diseases such as meningitis/encephalitis, there are at least 10 different bacteria and viruses that can cause the classic symptoms of fever, stiff neck, and altered mental status. Unfortunately, it is prohibitively expensive to test for all 10 organisms individually. As a result doctors are usually forced to treat patients empirically, without adequate knowledge of the true cause of disease. Empirical treatment is expensive, promotes antibiotic resistance, and puts patients at risk of adverse drug reactions. It may not even cover the rarer causes of disease. The present invention allows for the simultaneous detection of multiple genetic targets, such as genetic disease markers of a disease of interest, thereby allowing a physician to quickly, reliably and economically arrive at an accurate diagnosis.

The present invention may be provided as a diagnostic kit. A kit of the present invention may be tailored for the diagnosis of a variety of diseases and/or conditions such as meningitis/encephalitis, STDs, respiratory infections, etc. According to one embodiment of the present invention, a kit will contain PCR reaction tubes that are pre-loaded with a PCR reaction mixture. Such a kit may also include multiple target-specific primer pairs for the amplification and detection of genetic targets of interest. According to one embodiment of the invention, each tube will be ready for immediate use, and the laboratory technologist will simply add sample DNA and then run it using standard PCR equipment. Alternatively, a kit of the present invention may include some or all of the ingredients necessary to establish the suitable reaction conditions of the invention, such as a PCR reaction mixture, enzymes, and target-specific primers for example. A kit of the present invention may include predefined measures of the components for performing multiple repeats of the enhanced multiplex PCR method. Test controls may also be provided as a component of the kit of the present invention to confirm the reaction conditions of a given amplification reaction. For example, positive and negative

controls may be provided. In the case of a bacterial target, highly conserved targets like 16s rRNA and 23s rRNA genes may be employed. In the case of human molecular testing, human GAPDH or beta-globin genes may be used as positive test controls. These are highly conserved genes that can be used to confirm the presence of human DNA in a sample. Similarly, negative test controls may also be employed to provide indicators of the reaction conditions. A kit of the present invention preferably includes a set of instructions for practicing the method thereof. The kits of the present invention may be designed to be compatible with the major brands of PCR machines. Alternatively, the kits of the present invention may be tailored for a fully automated system specific to the present invention with integrated DNA extraction, and PCR amplification and detection functions.

The embodiments of the invention described above are intended to be exemplary only. The scope of the invention is therefore intended to be limited solely by the scope of the appended claims.

WE CLAIM:

1. A universal method for simultaneously amplifying greater than three genetic targets in a sample at equivalent amplification efficiencies, said method comprising:

   providing a primer pair specific to each of said greater than three genetic targets;

   adding said primer pairs to a PCR mixture;

   performing a series of PCR steps to amplify each of said multiple genetic sequences in said sample;

   wherein said PCR reaction mixture comprises said primer pairs at a final concentration of approximately 0.0025 μM – 0.05 μM per primer and Mg^{2+} at a concentration of approximately 5 – 12.5 mM in the reaction mixture.

2. The method of claim 1 wherein each of said primer pairs are provided to have a final concentration of approximately 0.01-0.02 μM in said reaction mixture.

3. The method of claim 1 wherein said PCR mixture comprises between 5.0 and 10 mM Mg^{2+}.

4. A method for preparing a PCR mixture for simultaneously amplifying greater than three genetic targets at equivalent amplification efficiencies, said method comprising:

   providing a PCR buffer or sterile water for dilution of PCR reactants;

   adding deoxyribonucleotides to the PCR buffer or water;

   adding Mg^{2+} to the PCR buffer or water such that the final concentration of Mg^{2+} in the PCR mixture is between 5.0 and 12.5 mM; and

adding a primer pair for each of said greater than three genetic targets to the PCR buffer or water such that the final concentration of each primer concentration to between 0.0025 and 0.05 μM.

5. The method of claim 4 wherein each of said primer pairs are provided such that the final concentration of each primer is approximately 0.01-0.02 μM in said PCR mixture.

6. The method of claim 4 between 5.0 and 10 mM.

7. A method for simultaneously detecting greater than three genetic targets in a sample, said method comprising:

providing a primer pair specific to each of said greater than three genetic targets;

adding said primer pairs to a PCR mixture;

performing a series of PCR steps to amplify each of said greater than three genetic sequences in said sample; and

detecting amplicons produced from the series of PCR steps,

wherein said PCR reaction mixture comprises said primer pairs at a final concentration of approximately 0.0025 μM – 0.05 μM per primer and Mg^{2+} at a concentration of approximately 5 – 12.5 mM in the reaction mixture.

8. The method of claim 7, wherein the amplicons are separated by agarose gel electrophoresis.

9. The method of claim 7, wherein each of said primer pairs are provided to have a final concentration in said PCR mixture of 0.005 – 0.05 μM.

10. The method of claim 7, wherein each of said primer pairs are provided to have a final concentration of approximately 0.01-0.02 μM in said PCR mixture.

11. The method of claim 7, wherein said PCR mixture comprises between 5.0 and 10 mM Mg^{2+}.

12. The method of claim 7, wherein said PCR mixture comprises approximately 7.5 mM Mg^{2+}.

13. The method of claim 1, wherein each of said greater than three genetic targets is a DNA sequence.

14. The method of claim 13, wherein said DNA sequences comprise bacterial DNA, viral DNA, plant DNA, animal DNA, fungal DNA or human DNA.

15. The method of claim 1, wherein each of said greater than three genetic targets is a RNA sequence and the method comprises the step of reverse transcribing the RNA sequences to produce cDNA before the PCR steps.

16. The method of claim 15, wherein the RNA sequences comprise bacterial RNA, viral RNA, plant RNA, animal RNA, fungal RNA or human RNA.

17. The method of claim 15, wherein the RNA sequences are mRNA.

18. The method of claim 7, wherein each of said greater than three genetic targets is a DNA sequence.

19. The method of claim 18, wherein the DNA sequences comprise bacterial DNA, viral DNA, plant DNA, animal DNA, fungal DNA or human DNA.

20. The method of claim 18, wherein the RNA sequences comprise bacterial RNA, viral RNA, plant RNA, animal RNA, fungal RNA or human RNA.

21. The method of claim 18, wherein the RNA sequences are mRNA.

22. A PCR mixture for use in simultaneously amplifying greater than three genetic targets at equivalent efficiencies, said mixture comprising a PCR buffer or water

containing dNTPs, 5.0 – 12.5 mM Mg^{2+} and a primer pair for each of said greater than three genetic targets at a concentration of 0.0025 – 0.05 μM,

wherein said PCR mixture in combination with a thermophilic DNA polymerase is suitable for simultaneously amplifying the greater than three genetic targets in a single reaction vessel without optimization.

23. The mixture of claim 22, wherein said PCR mixture includes dNTPs at a final concentration of approximately 0.25 mM each.

24. A kit for simultaneously amplifying greater than three genetic targets for detection, said kit comprising:

a PCR solution comprising Mg^{2+} and dNTPs; and

a primer solution comprising a primer pair corresponding to each of said greater than three genetic targets; and

a set of instructions for using contents of said kit to produce a PCR mixture for simultaneously amplifying the greater than three genetic targets in a sample to be tested;

wherein said PCR solution contains sufficient Mg^{2+} to produce a final concentration of 5.0 – 12.5 mM in the PCR mixture and said primer solution contains sufficient primers to produce a final concentration of 0.0025 – 0.05 μM of each primer in the PCR mixture.

25. The kit of claim 24, wherein said PCR solution is pre-loaded in at least one reaction vessel.

26. The kit of claim 25, wherein said at least one reaction vessel further includes said primer pairs.

27. The kit of claim 24, wherein said PCR solution and said primer solution are combined in a single PCR reagent solution.

28. The kit of any one of claims 24 – 27 further comprising a DNA polymerase enzyme.

29. A universal method for simultaneously amplifying greater than three genetic targets in a sample at equivalent amplification efficiencies, said method comprising:

providing a primer pair specific to each of said greater than three genetic targets;

adding said primer pairs to a PCR mixture;

performing a series of PCR steps to amplify each of said multiple genetic sequences in said sample;

wherein said PCR steps each comprise incubating the mixture for about 1 second at a denaturation temperature, followed by about 5 seconds at an annealing temperature and about 5 seconds at a product extension temperature, and

wherein said PCR reaction mixture comprises said primer pairs at a final concentration of approximately 0.0025 μM – 0.05 μM per primer and Mg^{2+} at a concentration of approximately 5 – 12.5 mM in the reaction mixture.

30. The method of claim 29, wherein the annealing temperature and the extension temperature are the same.

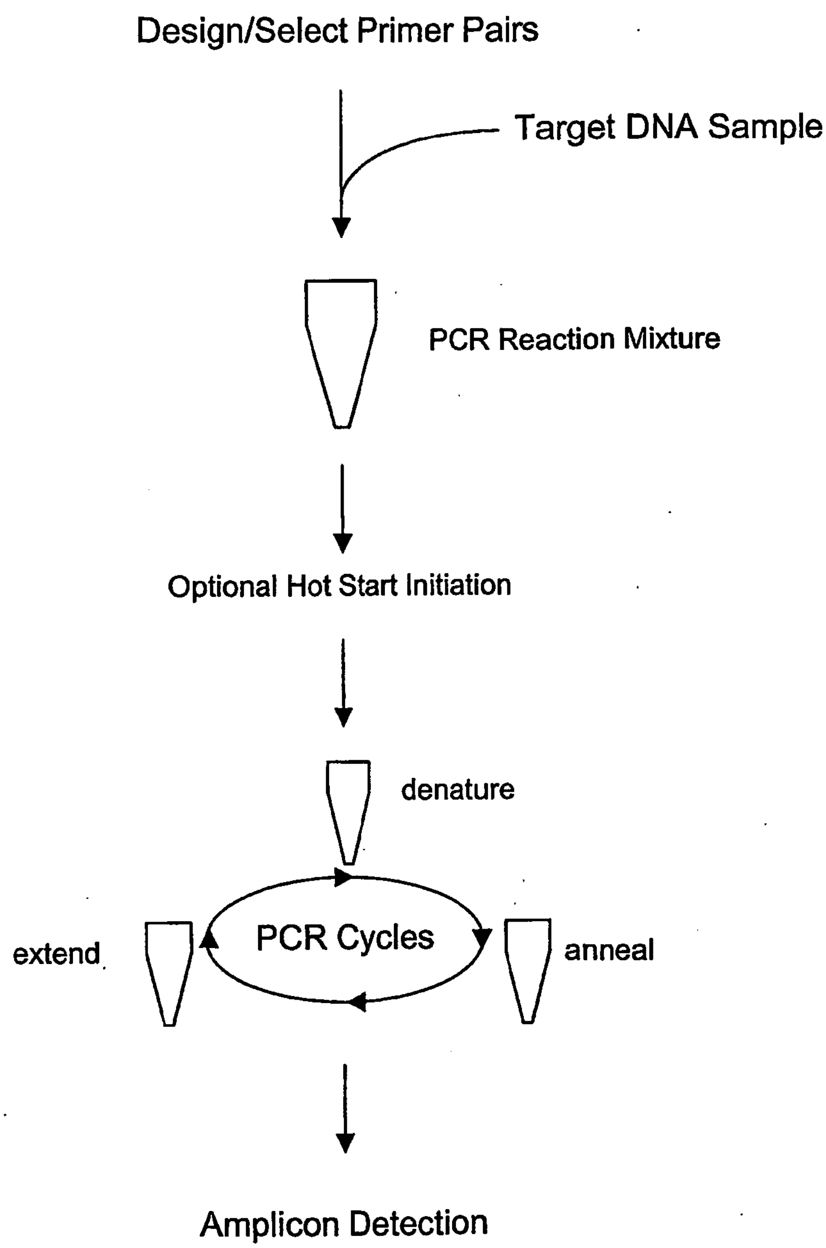

Design/Select Primer Pairs
Target DNA Sample
PCR Reaction Mixture
Optional Hot Start Initiation
denature
extend
PCR Cycles
anneal
Amplicon Detection

Figure 1

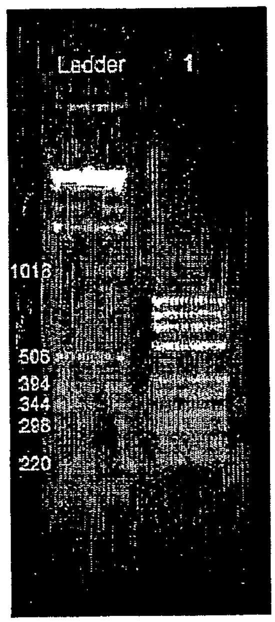
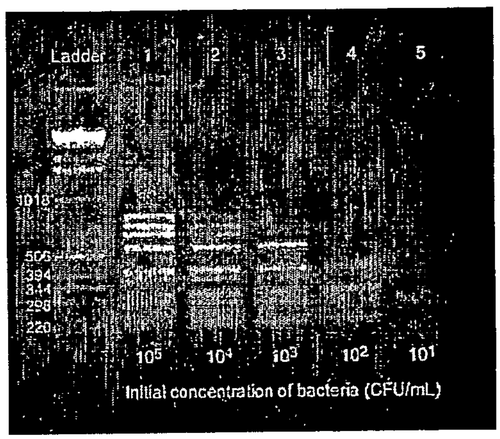


Figure 2


Ladder
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2
3
4
5
1018
506
394
344
298
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10^5
10^4
10^3
10^2
10^1
Initial concentration of bacteria (CFU/mL)

Figure 3

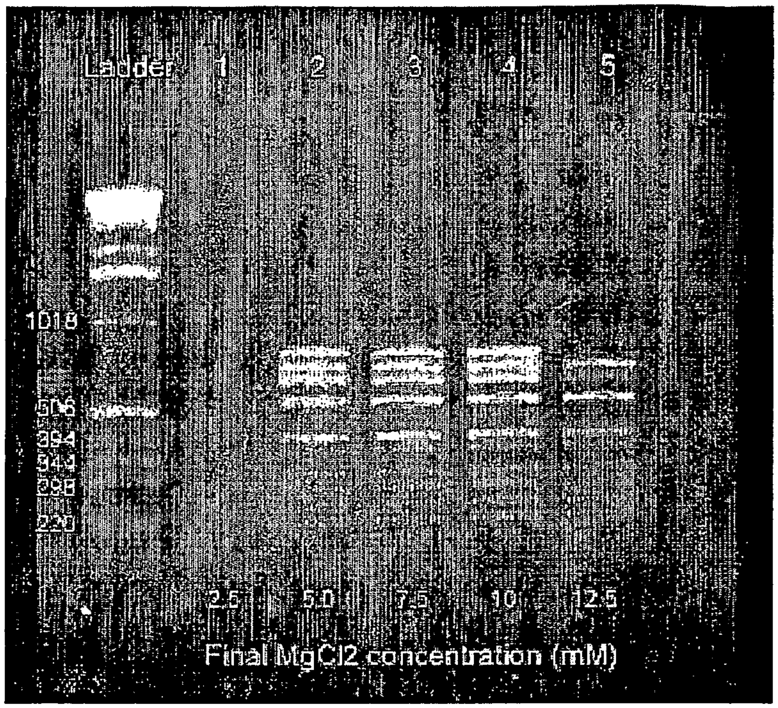
Ladder 1 2 3 4 5
1018
506
394
344
298
220
2.5 5.0 7.5 10 12.5
Final MgCl2 concentration (mM)

Figure 4

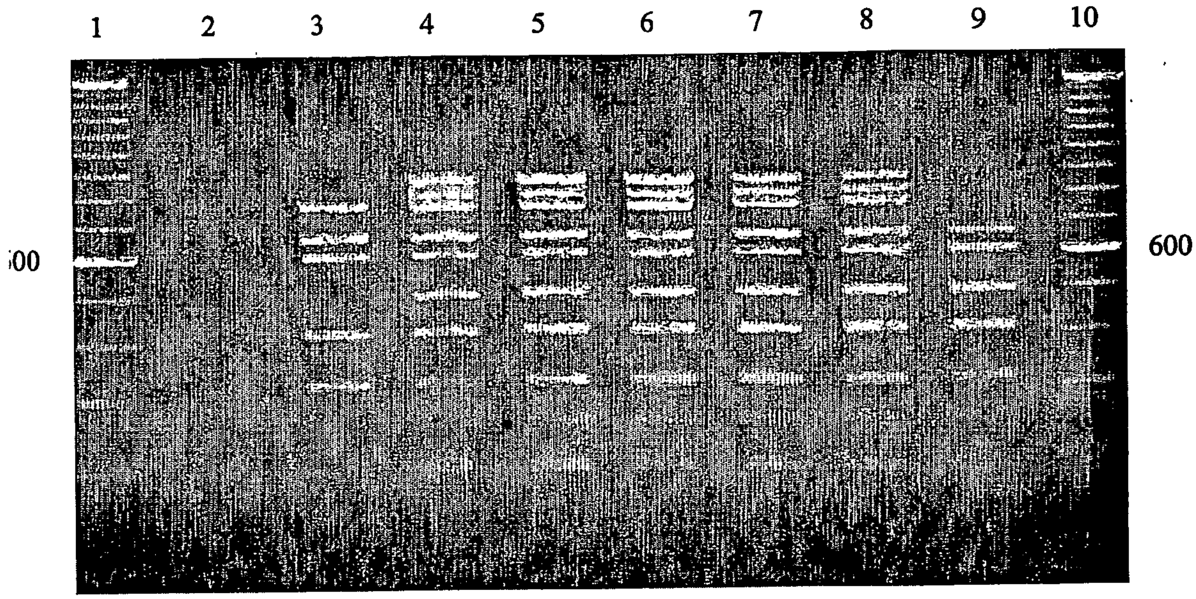

Figure 5

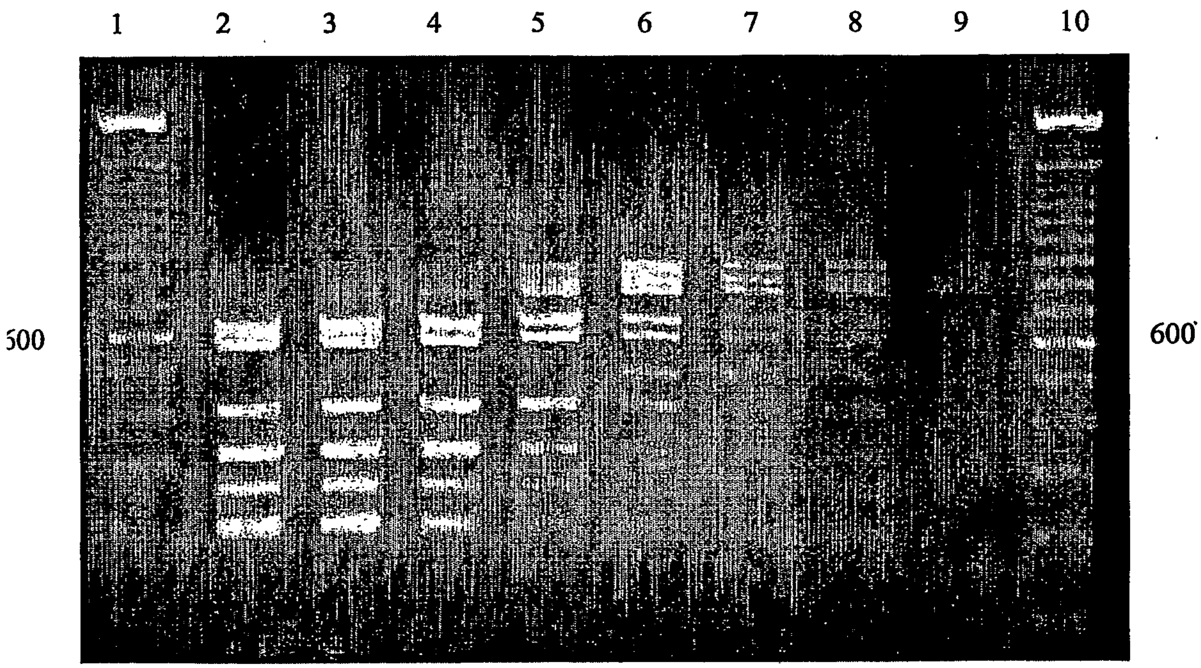

Figure 6

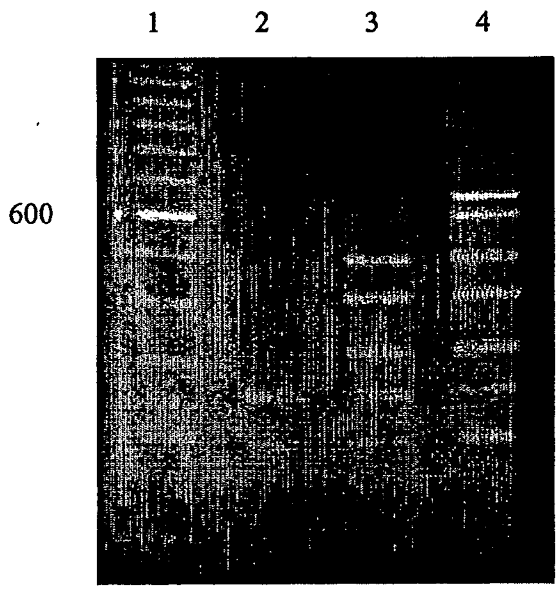

Figure 7

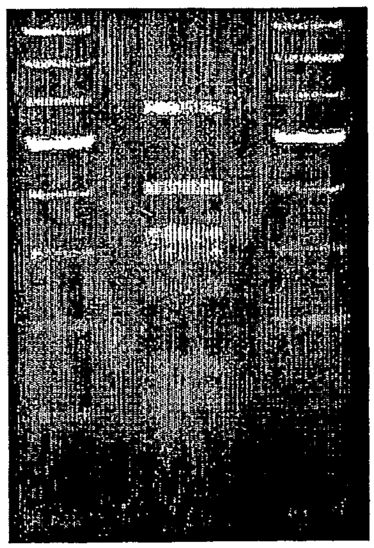

Figure 8

1 2 3 4 5 6

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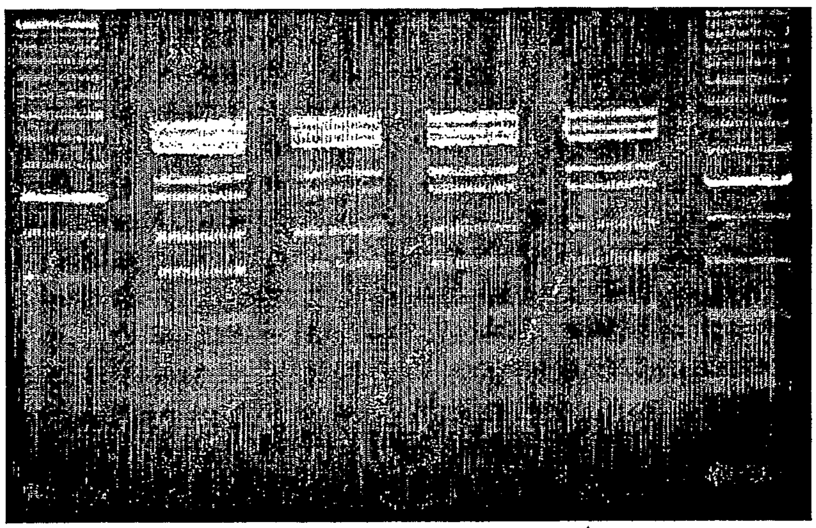


600

Figure 9

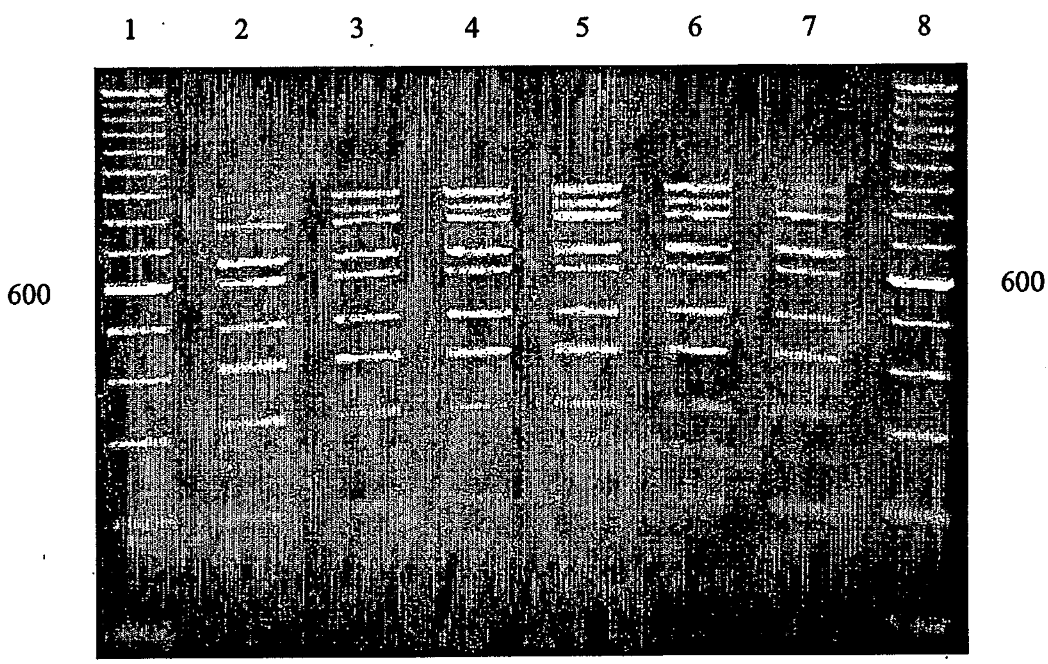


Figure 10

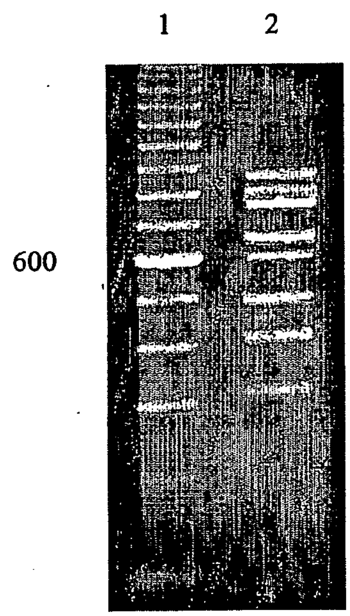


Figure 11

1 2

600

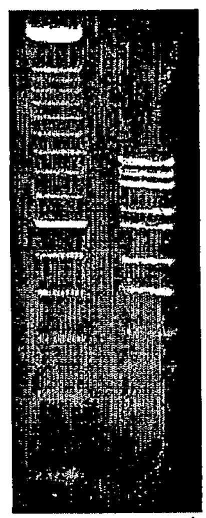


Figure 12

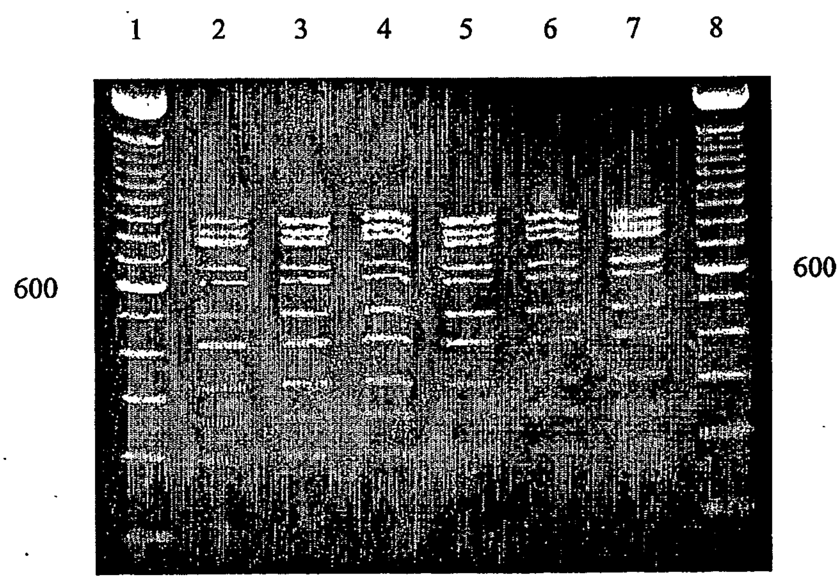


Figure 13

1 2

350

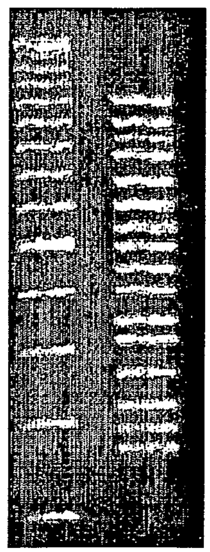


Figure 14

1 2 3 4 5 6 7 8

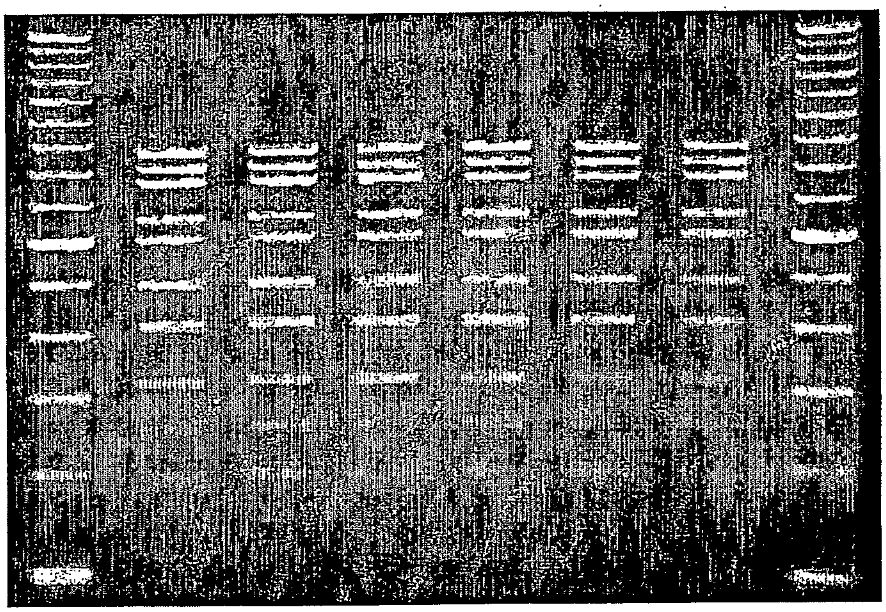
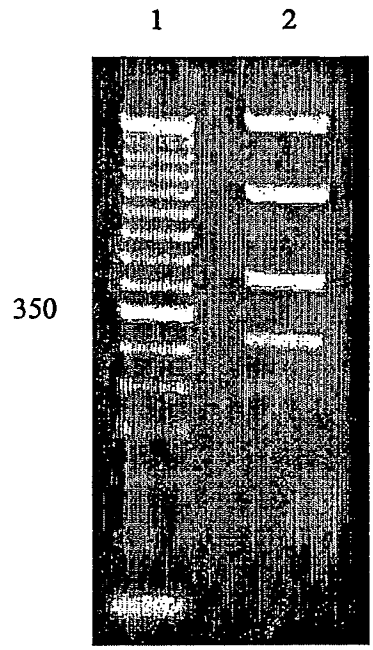
600  600

Figure 15

A



B

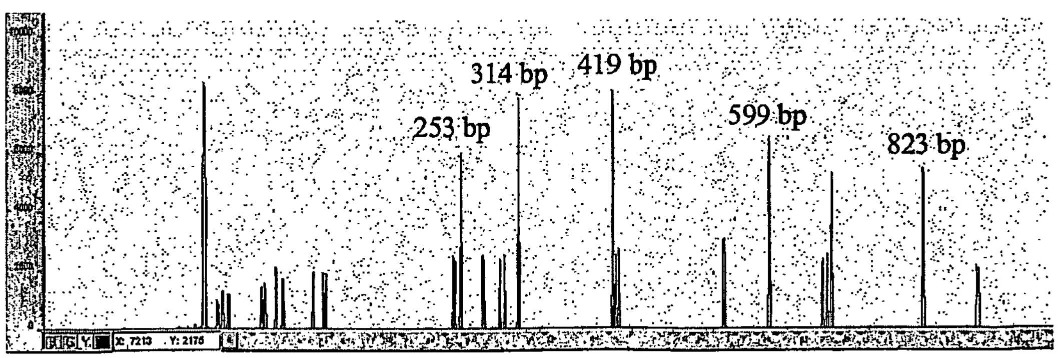


Figure 16

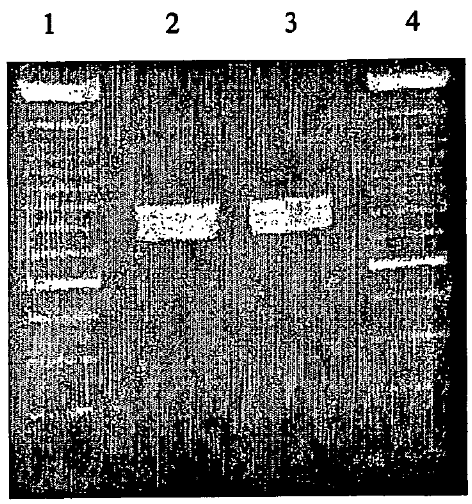


Figure 17

1 2 3 4

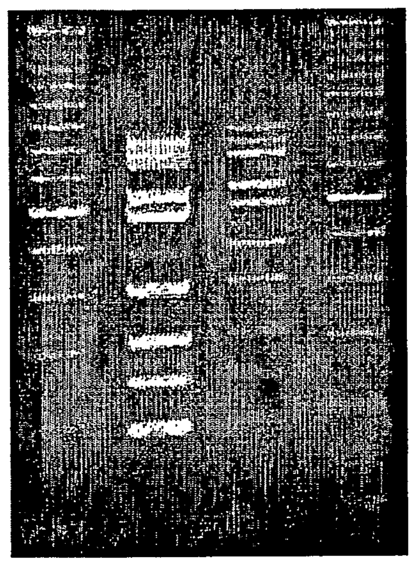


Figure 18

SEQUENCE LISTING

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<151> 2002-11-01

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 03/01681

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FISHBACK ANTHONY G ET AL: "Optimization of semi-automated microsatellite multiplex polymerase chain reaction systems for rainbow trout (Oncorhynchus mykiss)" AQUACULTURE, vol. 172, no. 3-4, 15 March 1999 (1999-03-15), pages 247-254, XP002270763 ISSN: 0044-8486 abstract page 250, paragraph 2; table 1 -----	1-30

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 February 2004	30.03.2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Favre, N

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LEM P ET AL: "Direct detection of mecA, nuc and 16S rRNA genes in BacT/Alert blood culture bottles." DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE. UNITED STATES NOV 2001, vol. 41, no. 3, November 2001 (2001-11), pages 165-168, XP002270764 ISSN: 0732-8893 cited in the application page 165, column 2, paragraph 4 - page 166, column 1, paragraph 1 -----	1-30
A	HENEGARIU O ET AL: "MULTIPLEX PCR: CRITICAL PARAMETERS AND STEP-BY-STEP PROTOCOL" BIOTECHNIQUES, EATON PUBLISHING, NATICK, US, vol. 23, no. 3, 1 September 1997 (1997-09-01), pages 504-511, XP000703350 ISSN: 0736-6205 cited in the application page 508, column 2, paragraph 2 - column 3, paragraph 1; figures 1,4 -----	1-30
A	SHUBER A P ET AL: "A SIMPLIFIED PROCEDURE FOR DEVELOPING MULTIPLEX PCRS" GENOME RESEARCH, COLD SPRING HARBOR LABORATORY PRESS, US, vol. 5, no. 5, 1 December 1995 (1995-12-01), pages 488-493, XP000546034 ISSN: 1088-9051 the whole document -----	1-30
A	MARKOULATOS P ET AL: "MULTIPLEX POLYMERASE CHAIN REACTION: A PRACTICAL APPROACH" JOURNAL OF CLINICAL LABORATORY ANALYSIS, NEW YORK, NY, US, vol. 16, no. 1, 2002, pages 47-51, XP009003351 cited in the application page 48, column 2, paragraph 4 -----	1-30
A	WALLIN JEANETTE M ET AL: "Constructing universal multiplex PCR systems for comparative genotyping." JOURNAL OF FORENSIC SCIENCES. UNITED STATES JAN 2002, vol. 47, no. 1, January 2002 (2002-01), pages 52-65, XP009026134 ISSN: 0022-1198 the whole document -----	1-30